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Transcriptional repression of *TWIST1* gene by Prospero-related homeobox 1 inhibits invasiveness of hepatocellular carcinoma cells

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ABSTRACT

Prospero-related homeobox 1 (PROX1) is important for liver development and down-regulation of this transcription factor in hepatocellular carcinoma (HCC) is associated with poor prognosis. We find that *PROX1* expression is inversely correlated with the expression of epithelial–mesenchymal regulator *TWIST1* in HCC cell lines and tumor tissues. We demonstrate that PROX1 directly binds to proximal promoter of *TWIST1* gene to repress its transcription and inhibits its downstream target gene *AKT2* expression which leads to reduction of cell migration and invasion. Moreover, PROX1 attenuates lung metastasis of HCC in vivo. These results support an anti-metastatic role of PROX1 via inhibiting *TWIST1*.

1. Introduction

Hepatocellular carcinoma (HCC), one of the most prevalent cancers in the world, consists of ~85% of primary liver cancers [1]. Patients with HCC have poor prognosis due to the high potential of recurrence and intra-hepatic metastasis [2]. Recent evidences suggest that epithelial to mesenchymal transition (EMT) may play an essential role in tumor metastasis. Several transcription factors such as SNAIL, TWIST1 and ZEB1/2 are key regulators of EMT and their expressions cause down-regulation of E-cadherin and other cell–cell junction proteins which leads to the gaining of mesenchymal traits and cancer cells become more aggressive and invasive [3,4]. Among these regulators, TWIST1 has been found to be over-expressed in HCC and is strongly associated with metastasis [5]. Subsequent studies demonstrated that TWIST1 increased migration, invasion and angiogenesis in this cancer [6,7]. More recently, Yang et al. provided evidence that TWIST1 may collaborate with SNAIL to promote HCC metastasis [8].

Prospero-related homeobox 1 (PROX1) was originally cloned as a homeobox gene which homologous to the *Drosophila prospero* gene and its expression was detected in lens, heart, liver, kidney, skeletal muscle, pancreas, and central nervous system [9,10]. Additionally, PROX1 has been shown to be critical for the development of liver and is thoroughly expressed from embryonic hepatoblasts to adult hepatocytes [11,12]. Recently, an elegant study reveals the potential role of PROX1 in hepatocytes by demonstrating that PROX1 interacts with estrogen receptor α and proliferator-activated receptor γ co-activator-1α to regulate the respiratory capacity and energy metabolism [13]. These results suggest that PROX1 is a physiological regulator of liver metabolic function. However, reduction of PROX1 was found in HCC and cholangiocellular carcinoma (CCC) and was associated with poor prognosis for HCC implicating a tumor suppressor role [14,15].

Whether PROX1 is involved in the process of HCC development is unclear. In this study, the effect of PROX1 on invasion of HCC cells was studied. We found that PROX1 directly repressed the transcription of an important EMT regulator *TWIST1* which led to down-regulation of expression of *AKT2*, a *TWIST1* target gene. Inhibition of TWIST1 and AKT2 protein reduced migration and invasion of HCC cells. In addition, we demonstrated that PROX1 suppressed lung metastasis of HCC in vivo. Our results provide a molecular basis for the anti-invasive and anti-metastatic action of PROX1.

2. Materials and methods

2.1. Cell culture and experimental reagents

Four HCC cell lines HepG2, Hep3B, Mahlavu and SK-HEP-1 were provided by Dr. Ming-Hong Tai (National Sun Yat-sen University, Kaohsiung, Taiwan). Flag-tagged PROX1 vector was a gift of You Hua Xie (Shanghai Institute for Biological Sciences, Shanghai,

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China). Constitutively active AKT (Myr-AKT) vector was provided by Dr. Min-Liang Kuo (National Taiwan University, Taipei, Taiwan). MYC-tagged TWIST1, HA-tagged AKT2, full-length *TWIST1* promoter and its subsequent truncation mutants were provided by Dr. Lu-Hai Wang (National Health Research Institutes, Miaoli, Taiwan). Anti-PROX1 antibody (07-537) was purchased from UpState (Charlottesville, VI) and anti-Actin antibody (MAB1501) was obtained from Millipore (Billerica, MA). Anti-TWIST1 (H81) and anti-MYC (9E11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-AKT2 (4057) antibody was obtained from New England Biolabs (Ipswich, MA). shRNAs were purchased from the National RNAi Core Facility of Academic Sinica (Taipei).

2.2. RNA isolation, reverse transcription-polymerase chain reaction and Western blotting

These assays were done as described previously [16]. The primer sequences used are shown in Supplementary Fig. 1.

2.3. Construction of mutant TWIST1 promoter-reporter plasmids

Full-length human *TWIST1* promoter containing −969 to +1 region and its deletion constructs were generated as described [17]. Mutations were introduced into the putative PROX1 binding sites of *TWIST1* promoter by the QuikChange Site-directed mutagenesis kit according to the procedures of manufacturer (Stratagene). The primer sequences used for mutagenesis are shown in Supplementary Fig. 2.

2.4. Promoter activity assay

Promoter activity of *TWIST1* gene was analyzed as described previously [17]. In brief, cells were plated onto 12-well plates at the density of 100,000 cells/well and grown overnight. The full-length, deletion or mutant promoter-luciferase constructs were transfected into cells with pcDNA (control), Flag-tagged PROX1 vector, luciferase (*Luc*) or *PROX1* shRNA. After transfection, cells were incubated in medium containing 10% FCS for 48 h and promoter activity was determined by using the Firefly luciferase assay system (Promega Corp.) and normalized for the concentration of cellular proteins. Results were evaluated by the Student's *t*-test and *p*-value <0.05 was considered significant.

2.5. Chromatin immunoprecipitation assay

pcDNA or Flag-tagged PROX1-transfected cells were fixed with 1% formaldehyde at 37 °C for 10 min. Cells were harvested and chromatin immunoprecipitation assay was done as described previously [18]. Anti-Flag or mouse IgG (as a negative control) were used for precipitating the protein/DNA complex. DNA fragments were recovered and subjected to PCR amplification by using the primers specific for the detection of −192 to −92 bp regions that contained the PROX1 binding site (−117/−111) in human *TWIST1* gene promoter. The sequences for the primers are forward: 5′-AATGGTTTGGGAGGACGAAT-3′, reverse: 5′-ACGTGAGGAGGAGGGACTTT-3′. DNA fragments were also quantified by using the real-time PCR detection system (Bio-Rad).

2.6. In vitro migration and invasion and experimental metastasis assay

In vitro migration and invasion assay and tail vein injection experimental metastasis experiments were performed as previously described [19,20]. Animal studies were carried out in nude mice and were approved by the Animal Care and Ethics Committee of the National Sun Yat-Sen University. Male

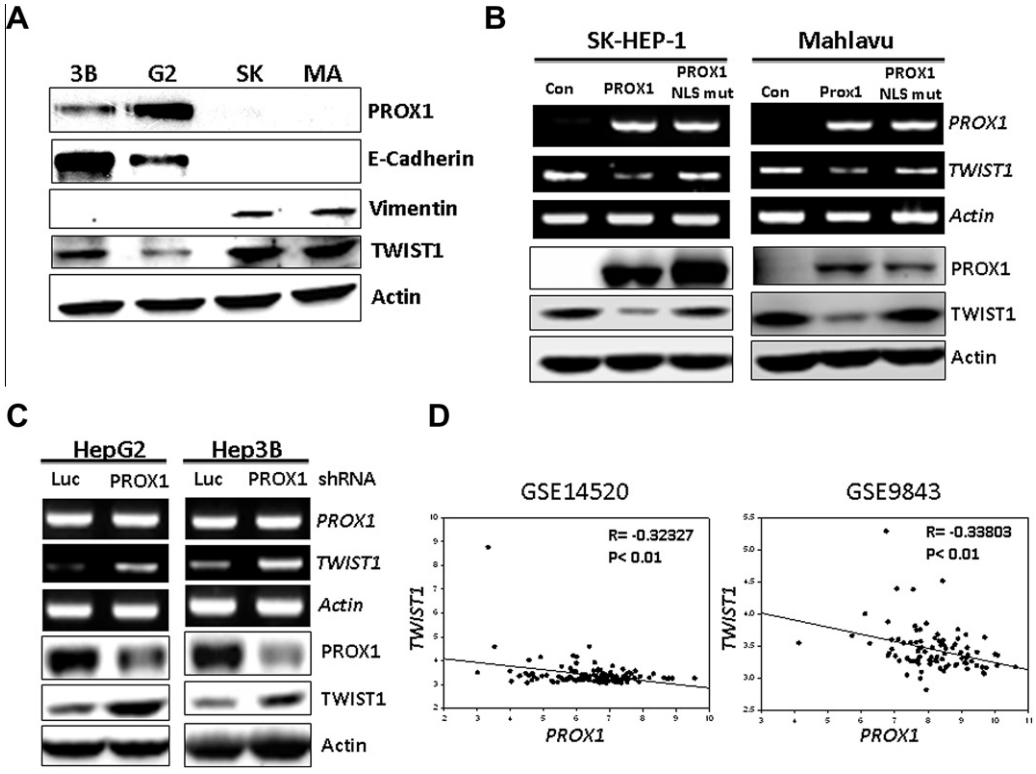

Fig. 1. PROX1 negatively regulates TWIST1 expression. (A) Expression of PROX1, TWIST1, E-cadherin and Vimentin in different HCC cell lines (3B: Hep3B; G2: HepG2; SK: SK-HEP-1; MA: Mahlavu). (B) SK-HEP-1 and Mahlavu cells were transfected with pcDNA, PROX1 or PROX1 NLS mutant which lacks nuclear localization signal expression vector. Expressions of *PROX1* and *TWIST1* mRNA and protein were determined. (C) HepG2 and Hep3B cells were transfected with luciferase (*Luc*, as a control) or *PROX1* shRNA for 48 h. *PROX1* and *TWIST1* mRNA and protein level were studied. (D) Correlation between *PROX1* and *TWIST1* were obtained from two independent datasets GSE14520 and GSE9843. Correlation coefficient and *p*-value were shown.

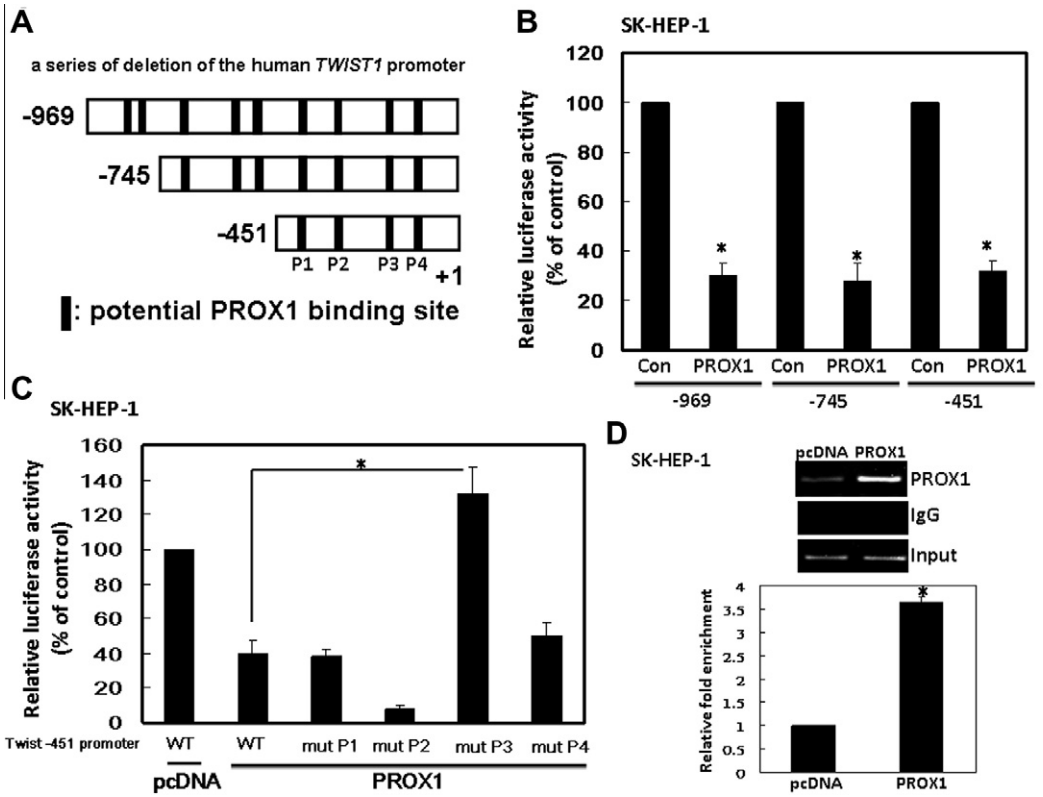

Fig. 2. PROX1 binds to *TWIST1* promoter. (A) Diagram showed the potential PROX1 binding sites in human *TWIST1* promoter and the deletion mutants used in this study. (B) Full-length and deletion mutants of human *TWIST1* promoter were co-transfected with pcDNA (Con) or PROX1 expression vector into SK-HEP-1 cells. After 48 h, cells were harvested for determination of luciferase activity and the promoter activity of cells co-transfected with pcDNA was defined as 100% $^{*}p < 0.05$ when compared to the control group. (C) The promoter-luciferase construct containing the −451/+1 *TWIST1* promoter region and mutants containing different mutated PROX1 sites were co-transfected with pcDNA or PROX1 expression vector into SK-HEP-1 cells. After 48 h, cells were harvested for determination of luciferase activity and the promoter activity of cells co-transfected with pcDNA and wild type −451/+1 twist promoter was defined as 100% $^{*}p < 0.05$. (D) Chromatin immunoprecipitation (ChIP) assay indicated the binding of PROX1 to *TWIST1* promoter was increased in PROX1-overexpreesing cells (top panel). Quantitative PCR was shown at bottom panel $^{*}p < 0.05$.

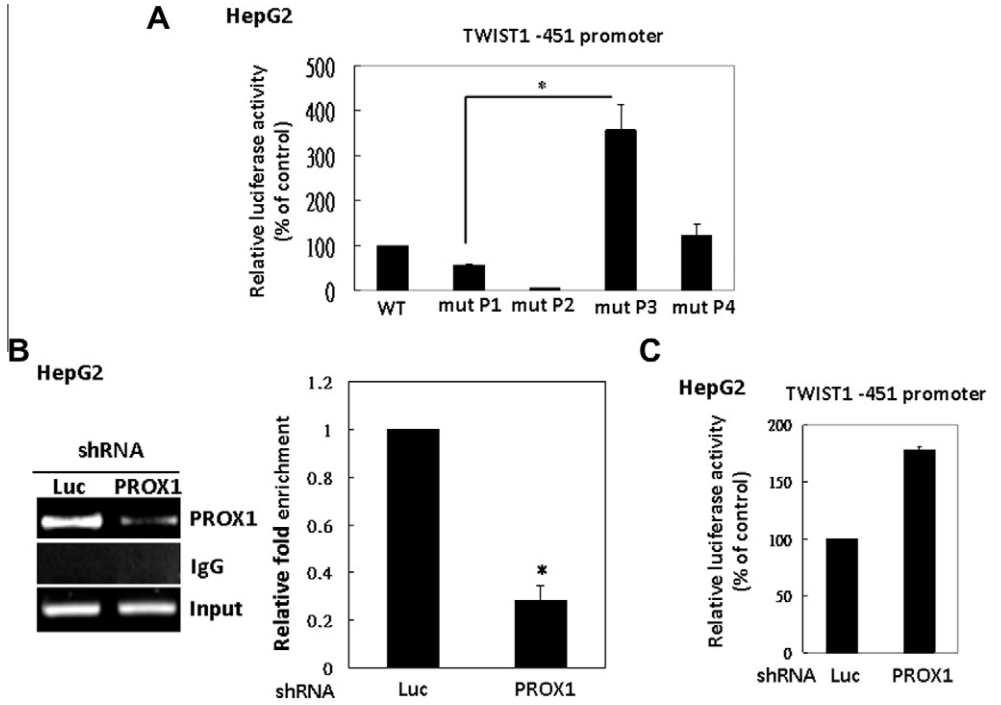

Fig. 3. Endogenous PROX1 binds *TWIST1* promoter and controls its transcription. (A) The promoter-luciferase construct containing the −451/+1 *TWIST1* promoter region (WT) and mutants containing different mutated PROX1 sites was transfected into HepG2 cells that expressed high level of endogenous PROX1 protein and the promoter activity was determined. (B) ChIP assay was conducted to study the binding of PROX1 to *TWIST1* promoter (left panel). Quantitative PCR was shown at right panel $^{*}p < 0.05$. (C) The −451/+1 *TWIST1* promoter-luciferase construct was transfected into HepG2 cells expressing luciferase (*Luc*) or *PROX1* shRNA and the promoter activity was determined.

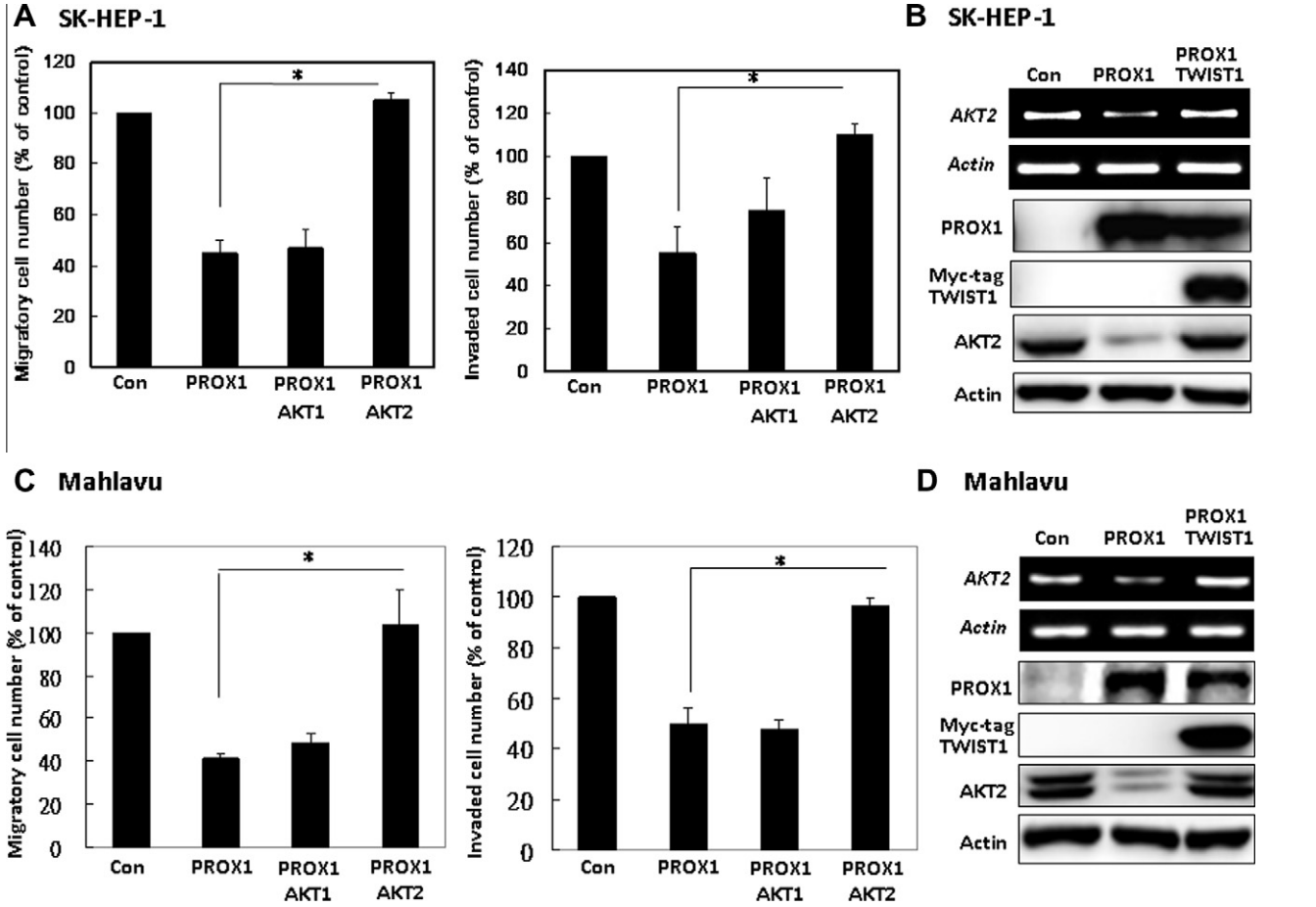

Fig. 4. TWIST1 reverses PROX1-inhibited migration and invasion via AKT2. (A) SK-HEP-1 cells were transfected with control (Con), PROX1, AKT1 or AKT2 expression vectors. Cells were subjected to migration and invasion study by using transwell assays. The number of migratory or invasive cells of the control group was defined as 100% $^{*}p < 0.05$, when compared to the control group. (B) *AKT2* mRNA and protein expression of these cells were studied. (C) Mahlavu cells were transfected with control (Con), PROX1 or TWIST1 expression vectors and cells were subjected to migration and invasion study described in (A). (D) *AKT2* mRNA and protein expression of Mahlavu cells transfected with different vectors were investigated.

BALB/cAnN-Foxn1 nude mice (5 weeks old) were obtained from National Laboratory Animal Center of National Science Council (Taipei, Taiwan). Mice were randomly divided into two groups (n = 5 for each group). pcDNA or Flag-tagged PROX1-transfected SK-HEP-1 cells (5×10^5 cells/mice) were injected via tail vein (day 0). Fifteen days after cell injection, animals were killed. Lungs of the experimental animals were stained with a contrast medium (15% India ink in distilled water) by injecting the medium into the trachea and then fixed in a fixation solution (100 ml of 70% alcohol, 10 ml of formaldehyde, and 5 ml of glacial acetic acid). The number of surface tumors was counted.

2.7. Oncomine data analysis

Oncomine (http://www.oncomine.org) is a cancer microarray database and integrated data-mining platform. Two representative and independent HCC microarray data sets GSE14520 (sample size = 130) and GSE9843 (sample size = 91) were used to analyze the correlation between *PROX1* and *TWIST1* expression. The correlation coefficient and *p*-value were evaluated using PROX1/207401_at and TWIST1/213943_at, from GSE14520 to GSE9843, respectively, by statistic program R (a free software environment for statistical computing and graphics which could be found at http://www.r-project.org).

3. Results

3.1. PROX1 is a negative regulator of TWIST1

Expression of PROX1 was screened in various HCC cell lines. Our data showed that well-differentiated (epithelial phenotype) HepG2 and Hep3B, but not poorly-differentiated (mesenchymal phenotype) SK-HEP-1 and Mahlavu cells expressed PROX1 protein (Fig. 1A). Comparison of the epithelial–mesenchymal transition (EMT)-related markers in these cell lines revealed that E-cadherin was detectable in HepG2 and Hep3B cells while it was absent in SK-HEP-1 and Mahlavu cells. Conversely, high levels of vimentin and TWIST1 protein were found in SK-HEP-1 and Mahlavu cells while these two mesenchymal markers were undetectable or very low in PROX1-positive HepG2 and Hep3B cells (Fig. 1A). Because TWIST1 has been demonstrated to play a critical role in the induction of EMT and invasion of HCCs, we tested whether PROX1 could affect *TWIST1* expression. Ectopic expression of PROX1 in SK-HEP-1 and Mahlava cells led to down-regulation of *TWIST1* mRNA and protein (Fig. 1B). This effect was not observed in cells expressed a PROX1 mutant protein which could not enter nucleus due to the mutation of nuclear localization signal (NLS) at the N-terminal (9–14 a.a.). On the contrary, knockdown of PROX1 by shRNA in HepG2 and Hep3B cells caused up-regulation of *TWIST1* mRNA and protein (Fig. 1C). These data suggested that PROX1 functions

as a negative regulator of *TWIST1*. To confirm the association between *PROX1* and *TWIST1* expression, we examined microarray data from patient samples contained within the Oncomine database. Indeed, two independent data sets GSE14520 (sample size = 130) and GSE9843 (sample size = 91) showed a significant inverse correlation between the mRNA level of these two genes (Fig. 1D). Both cell-based and tissue microarray data suggested that PROX1 could negatively regulate *TWIST1*.

3.2. PROX1 down-regulates TWIST1 via transcriptional repression

Two lines of evidences suggested that *TWIST1* may be a direct transcriptional target of PROX1. Firstly, expression of PROX1 reduced *TWIST1* mRNA level while knockdown of PROX1 caused up-regulation of *TWIST1* mRNA. Secondly, the NLS mutant PROX1 protein could not repress *TWIST1* expression because it could not enter nucleus and bind DNA. Two previously reported *Perspero* binding consensus sequences, C[A/T][C/T]NNC[T/C] and CGTCT[T/A] [21,22], were used to blast the human *TWIST1* gene promoter region. Our results showed that 9 potential PROX1 binding sequences were identified between −969 and +1 region of human *TWIST1* promoter (Fig. 2A). Deletion of the *TWIST1* promoter region from −969 to −451 bp did not affect the inhibition of promoter activity by PROX1 suggesting the response elements are located at the −451/+1 region (Fig. 2B). Four potential PROX1 binding sites located within this region were mutated and we found that only mutation of the third site (mut-P3) located at −117/−111 bp region effectively reversed PROX1-induced inhibition (Fig. 2C). ChIP assay clearly demonstrated that PROX1 bound to *TWIST1* promoter which contained P3 site and this binding was specific because immunoprecipitation with non-immune immunoglobulin did not yield any signals (Fig. 2D). Quantification of the immunoprecipitated DNA fragments by quantitative PCR revealed that a 3.5-fold increase of PROX1 binding was detected (Fig. 2D). This conclusion was further confirmed in HepG2 cells which expressed high endogenous PROX1. As shown in Fig. 3A, mutation of the P3 site reversed the inhibition of *TWIST1* promoter activity by endogenous Prox1. ChIP assay verified the binding of endogenous PROX1 to *TWIST1* promoter and knockdown of PROX1 reduced this binding by 70% (Fig. 3B). Additionally, attenuation of PROX1 promoter binding was associated with increase of *TWIST1* promoter activity (Fig. 3C). By manipulation of PROX1 level by over-expression or shRNA silencing, we verified that PROX1 directly binds to *TWIST1* promoter to repress its transcription.

3.3. PROX1 inhibits migration and invasion via inhibiting TWIST1-induced AKT2

Effect of PROX1 on aggressive behavior of HCC cells was tested. We found that expression of PROX1 reduced cell migration and invasion in poorly-differentiated SK-HEP-1 (Fig. 4A). Because Twist is a transcription factor, it needs to turn on specific target genes to promote HCC cell invasion. Lines of evidences suggest that AKT2 is a potential candidate. Firstly, *AKT2*, but not *AKT1*, gene has been found to be a direct transcriptional target of TWIST1 [23]. Secondly, AKT2 over-expression was found in HCC and was strongly correlated with poor prognosis [24]. Thirdly, AKT2 is expressed higher than AKT1 in HCC cell lines [25]. In consistent with our hypothesis,

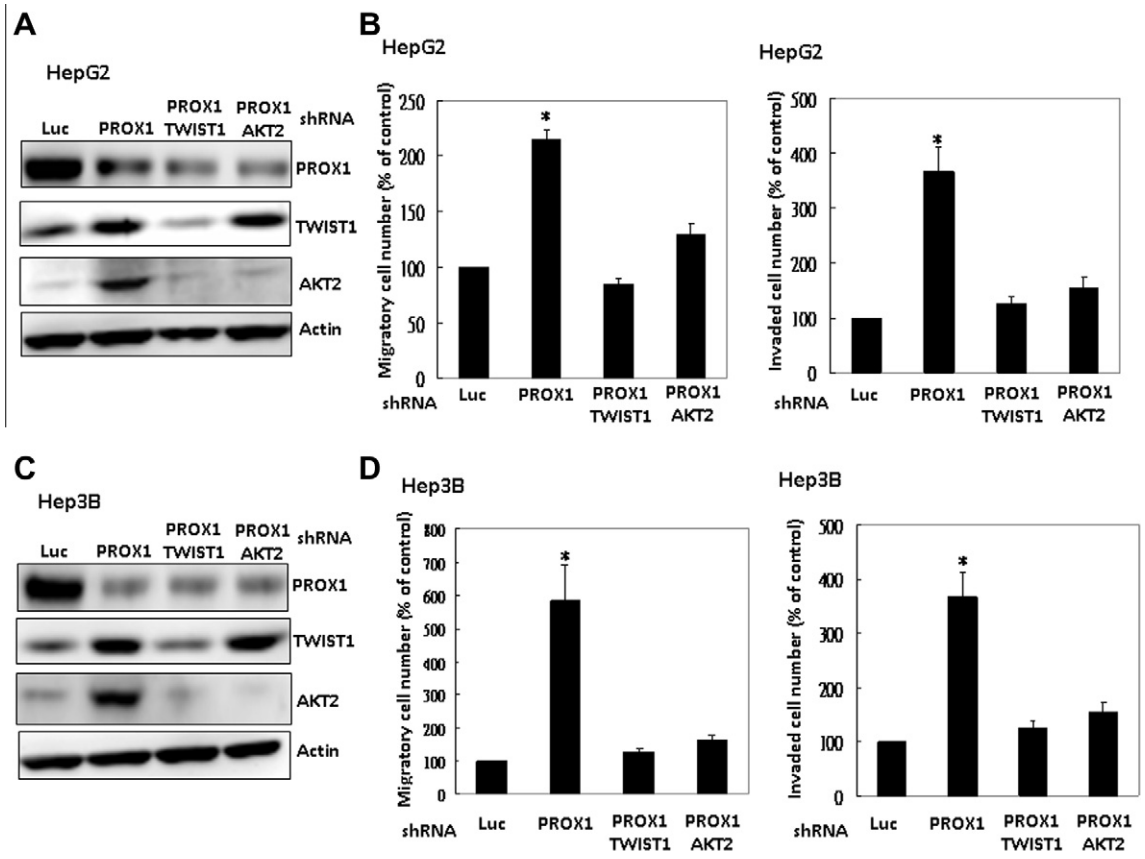

Fig. 5. AKT2 is critical for TWIST1 to reverse PROX1-inhibited migration and invasion. (A) HepG2 cells were transfected with luciferase (*Luc*), *PROX1*, *TWIST1* or *AKT2* shRNA. Expression of these proteins was investigated by Western blotting. (B) These cells were also subjected to migration and invasion assays. The number of migratory or invasive cells of the control group (*Luc*) was defined as 100% $^{*}p < 0.05$ when compared to the control group. (C) Hep3B cells were transfected with various shRNA and protein expression of these proteins was investigated by Western blotting. (D) Migration and invasion of these cells were studied.

ectopic expression of AKT2, but not AKT1, fully counteracted the inhibition of migration and invasion by PROX1 in SK-HEP-1 cells (Fig. 4A). In addition, enforced expression of PROX1 reduced *AKT2* mRNA and protein in SK-HEP-1 cells which could be reversed by TWIST1 (Fig. 4B). Similar results were obtained in Mahlavu cells indicating this is not a cell line-specific effect (Fig. 4C and D). Conversely, knockdown of endogenous PROX1 increased TWIST1 and AKT2 protein level in HepG2 cells (Fig. 5A). In addition, knockdown of TWIST1 abolished AKT2 protein up-regulation induced by PROX1 inhibition suggesting TWIST1 acts as an upstream regulator of AKT2. Migration and invasion of HepG2 cells were increased after knocking down of PROX1 (Fig. 5B). When TWIST1 or AKT2 was simultaneously inhibited by shRNA, this increase was significantly repressed. Similar results were also obtained in Hep3B cells (Fig. 5C and D). It should be noted that TWIST1 and AKT2 shRNA did not affect basal migratory and invasive ability of HepG2 and Hep3B cells because these cells expressed few TWIST1 and AKT2 proteins (Supplementary Fig. 3). After knockdown of PROX1, TWIST1 and AKT2 levels significantly went up and cell migration/invasion was enhanced. Then, these cells became sensitive to TWIST1 and AKT2 shRNA indicating the inhibitory effect of these two shRNAs is specific. Our data suggested that PROX1 inhibits migration and invasion of HCC cells via suppressing the TWIST1/AKT2 axis.

3.4. Inhibition of lung metastasis by Prox1 in vivo

Anti-metastatic effect of PROX1 was also tested in vivo. Control- and PROX1-expressing SK-HEP-1 cells were injected via tail vein and lung metastatic nodules were checked at 15 days after injection. Our data showed that number of metastatic nodules on lung surfaces was dramatically reduced by 80–85% in PROX1-expressing group (Supplementary Fig. 4).

4. Discussion

Although PROX1 has been suggested to play a tumor suppressive role in a variety of cancers, including HCC, CCC, hematologic malignancies, breast cancer, and pancreatic cancer [14,15,26,27], the detailed anti-cancer mechanism is still obscure. Our study provides evidence that PROX1 directly inhibits *TWIST1* gene expression which causes AKT2 down-regulation and consequently prevents metastatic colonization of HCC cells.

TWIST1 is an important oncogene and EMT regulator which promotes cancer progression via different mechanisms. One of the first TWIST1 transcriptional targets identified is E-cadherin [28]. Twist directly binds to E-cadherin promoter to repress its expression. Down-regulation of E-cadherin subsequently attenuates cell-cell adhesion and enhances cell migration and invasion. Increase of angiogenesis also is involved in TWIST1-promoted cancer progression because TWIST1 enhances production of vascular endothelial growth factors in breast cancer [29]. Because TWIST1 is up-regulated in many cancers, it seems to be a potential target for cancer therapy. However, the mechanism of TWIST1 overexpression in cancer cells is still unclear and it may be cancer type-dependent. In this study, we demonstrate that TWIST1 is a transcriptional repression target of PROX1 because PROX1 binds TWIST1 promoter in vivo (as shown by ChIP assay) and represses TWIST1 promoter activity via the potential binding site located at the −117/−111 bp of the promoter region. In addition, we identify a downstream mediator AKT2 which mediates PROX1-induced metastatic inhibition. Our results provide the first molecular basis to explain the anti-metastatic action of PROX1 in HCC cells.

The regulation of AKT2 by PROX1 is a critical finding. By using transgenic models, it is found that different isoforms of AKT play distinct roles in mammary tumor progression [30,31]. The authors conclude that AKT1 is important for cancer induction (or initiation) and AKT2 is primarily involved in metastatic dissemination. Similarly, AKT2 plays a critical role in the establishment of colorectal cancer metastasis [32]. However, overexpression of AKT1 could not restore metastatic potential in cells with down-regulated AKT2 indicating non-redundant roles for individual AKT isoforms and emphasize the specific function of AKT2 in metastasis. The unique role of AKT2 is also verified by this study which demonstrates that only AKT2 could rescue PROX1-inhibited migration and invasion. Because tail vein injection model mainly assesses the steps of extravasion, adhesion and colonization, further experiments will be needed to clarify the detailed mechanism of AKT2-mediated metastasis of HCC cells.

In summary, we demonstrate that PROX1 is a metastatic repressor in HCC cells and identify TWIST1 as a major target of PROX1 to attenuate metastatic signaling through AKT2. This finding provides new insight for HCC metastasis and may be helpful for the development of new strategy for treatment and prevention.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.08.034.

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